REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
06-11-2007	Final Report	15-Sep-2006 - 14-Sep-2007

4. TITLE AND SUBTITLE
New materials based on spider silk

5a. CONTRACT NUMBER
W911NF-06-1-0451

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
Scheibel

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Technische Universitat Munchen
Technische Universitat Munchen
D-80290
00000 -

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
49686-LS.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for public release; Federal purpose rights

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
In this project, we investigated the dragline silk proteins ADF-3 and ADF-4 of the spider Araneus diadematus. Adf-3 and Adf-4 cDNA can each be expressed directly in insect cells using the Baculovirus-expression-System. Recombinant ADF-4 is insolubly produced in the cytoplasm of insect cells and soluble produced upon secretion into the media. Further, parameters influencing stability and solubility of recombinantly produced ADF analogues have been investigated. We detected that intermolecular disulfide bridges formed between monomers of the ADF analogues stabilized formed dimer. Additionally, it was found that potassium phosphate triggers assembly of ADF analogues into spheres and fibrils.

15. SUBJECT TERMS
spider silk

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Thomas Scheibel
U	U	U	SAR		19b. TELEPHONE NUMBER 499-215-5710

Report Title

New materials based on spider silk

ABSTRACT

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List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Vendrely, C. and Scheibel, T. (2007). Biotechnological production of spider silk proteins enables new applications. Macromolecular Biosciences, 7, 401-409.

Number of Papers published in peer-reviewed journals: 1.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Vendrely, C., Exler, J. and Scheibel, T. (2007). Role of individual spider silk domains during assembly.
Poster at the conference on "Protein Assembly in Materials, Biology and Medicine", June 2007 on Crete, Greece

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 1

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Number of Inventions:

Graduate Students

NAME	PERCENT SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Charlotte Vendrely	1.00
FTE Equivalent:	**1.00**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

New materials based on spider silk

Final report

ARO W911NF-06-1-0451

I) Table of content

II) List of appendixes, illustrations, and tables

III) Statement of the problem studied

Spider silks are considered as one of the most resilient materials in nature. Their outstanding properties exhibit a combination of high strength and good elasticity. Spider silks are made of high molecular weight proteins characterized by a long core sequence built of repetitive motives and flanked by two short non repetitive domains. We have successfully developed a method for recombinantly expressing *adf-3* and *adf-4*, spider silk genes obtained from a cDNA library from the garden spider *Araneus diadematus*, using insect cells as expression hosts. In order to take advantage of spider silks for applications as biomaterials, we proposed to analyze the solubility, structure, stability, posttranslational modifications, and assembly properties of rADF-3 and rADF-4 recombinantly produced in insect cells.

IV) Summary of the most important results

1) Production of rADF-3 and rADF-4 in insect cells

a) Expression in insect cells

Using the baculovirus expression system, ADF-3 and ADF-4 can be produced in the cytoplasm and in the media of insect cells. Partial cDNAs coding for the known carboxy-terminal sequences of ADF-3 and ADF-4 have been cloned into the baculoviral genome and recombinant virus was used to infect different insect cell lines. Two strategies have been followed (Vendrely and Scheibel, 2007):

- in the first strategy, recombinant virus was used to infect *sf*9 cells from the fall armyworm *Spodoptera frugiperda*. The proteins were produced in cell cytoplasm and rADF-3 remained soluble, while rADF-4 assembled into fibers (figure 1a) (Huemmerich et al., 2004a).
- in the second strategy, proteins were secreted into the insect media (figure 1b). To enable secretion, the short sequence encoding the honey bee secretion signal was (genetically) fused to the spider silk protein. A respective recombinant virus was used to infect *high five* cells of the cabbage looper *Trichopulsia ni*. This cell line was chosen for their good propensity to secrete recombinant proteins. Secreted rADF-4 was soluble indicating that secretion, *i.e*. possible post-translational modifications, can modify its solubility.

A B

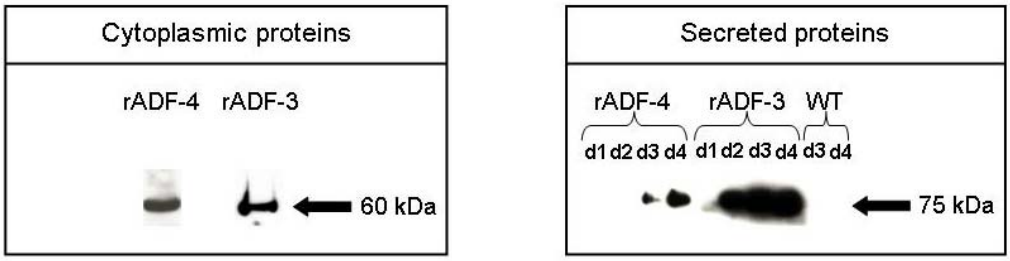

Figure 1. Recombinant production of ADF-4 and ADF-3 in insect cells. A: Western Blot analysis of *sf*9 insect cells lysate after cytoplamic production of rADF-4 and rADF-3. B: Western Blot analysis of growth media after secreted production in *High Five* cells for 1 (d1), 2 (d2), 3 (d3), or 4 (d4) days after infection (Vendrely and Scheibel, 2007).

b) Purification

- Purification of cytoplasmic rADF-4

We took advantage of the insolubility and stability of rADF-4 to elaborate a purification strategy without chromatographical methods (Huemmerich et al., 2004a). *Sf*9 cells expressing rADF-4 have been collected 4 days post-infection with recombinant virus. Cells have been resuspended in lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 15 mM $MgCl_2$, 1 % (v/v) triton X-100). Aggregates were sedimented by centrifugation at 5 000 g for 5 min and the pellet washed with 8M urea. rADF-4, included in the pellet, was solubilized in 6 M guanidinium thiocyanate. Refolding of rADF-4 has been successfully achieved by dilution into 10 mM Tris pH 8, 0.5M arginine.

- Purification of secreted rADF-3

As secreted rADF-3 is produced in larger amounts than rADF-4, we first started to establish a purification strategy for this protein. Both secreted spider silk proteins include an amino-terminal His-tag which allows purification by chromatography on Ni^{2+}-affinity column. Purification has been performed on a Ni^{2+}-Sepharose 6 Fast Flow column with 20 mM HEPES pH 8, 0.5 M NaCl, 5 mM Imidazole, 8M urea as binding and wash buffers and 20 mM HEPS pH 8, 0.5 M NaCl, 1 M Imidazole, 8M urea as elution buffer (figure 2).

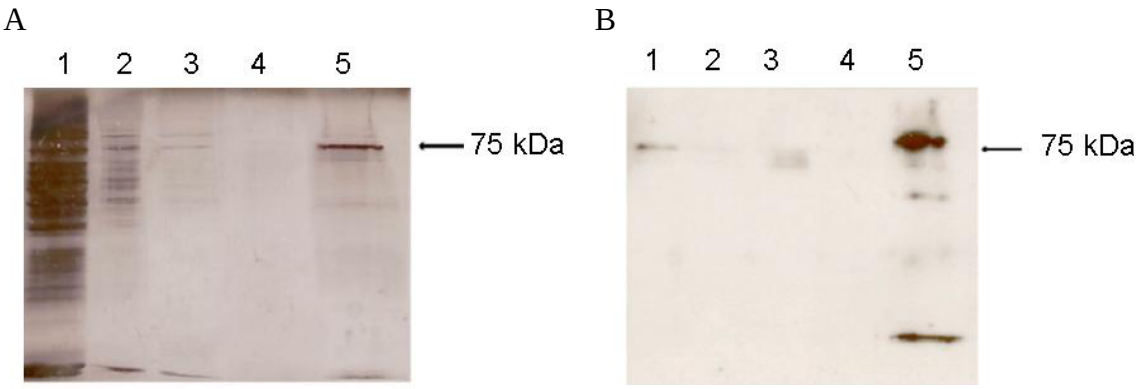

Figure 2. Purification of secreted rADF-3 from the medium of insect cells culture on Ni^{2+} affinity column. A: silver stained 10% SDS-PAGE analysis. B: Western Blot analysis. (1) proteins loaded on the column. (2) 1:5 dilution of protein loaded on the column. (3) flow through of the column. (4) proteins washed from the column. (5) rADF-3 eluted from the column.

2) Stability and assembly of eADF-3

Since establishing the secretion and purification protocol for rADF-3 was more time-consuming than expected, we started to study an engineered silk analogue eADF-3 produced in *Escherichia coli* in a parallel approach. Information gained from the investigation of the parameters influencing the structure, stability and assembly of eADF-3, will help to further characterize rADF-3 produced in insect cells.

a) Production of eADF-3

Previously, a strategy to produce repetitive spider silk proteins in *Escherichia coli* has been established in our laboratory (Huemmerich et al., 2004b; Vendrely and Scheibel, 2007). eADF-3 consists of a repetition of alanin-rich motives (named “A”) and glutamine-rich motives (named “Q”). The short carboxy-terminal domain of the protein shows a non repetitive (NR) unique sequence. eADF-3 is dimeric and the NR domain contains a cystein which forms an intermolecular disulfide bridge between the two monomers. In this study, we employed three different eADF-3 variants: $AQ_{12}NR$, composed of a repetition of 12 AQ motives and the NR terminus; $AQ_{24}NR$, composed of a repetition of 24 AQ motives and the NR terminus and AQ_{24}, composed of a repetition of 24 AQ motives without NR terminus.

b) Thermal stability of eADF-3

The secondary structure of eADF-3 was analyzed by circular dichroism (CD) spectroscopy (figure 3). Typically, CD spectrum obtained from eADF-3 show a random coil structure with some α-helix content originating from the NR domain (Huemmerich et al., 2004b). The

repetitive part of $AQ_{24}NR$ is twice as long as the one of $AQ_{12}NR$, revealing a higher content in random coil and a CD spectrum with a higher signal at 205 nm (figure 3).

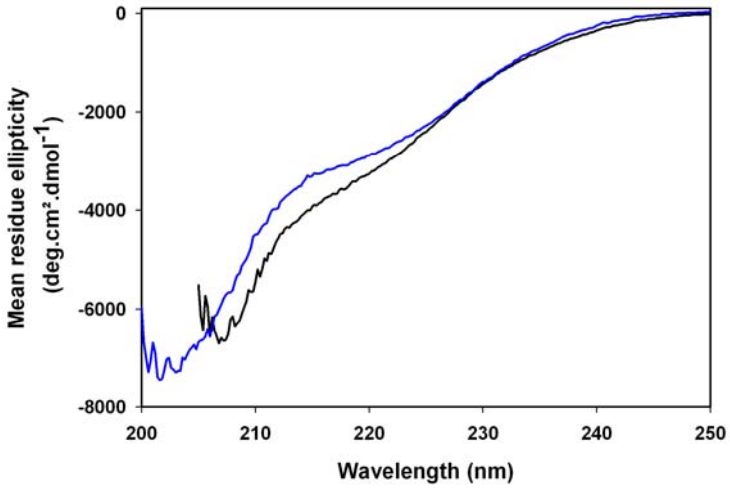

Figure 3. Circular dichroism spectra of $AQ_{24}NR$ (black) and $AQ_{12}NR$ (blue).

Thermal stability of eADF-3s has been investigated using thermal denaturation monitored by CD (figure 4). The influence of the NR domain on the stability of the protein has been examined in reducing conditions. Thermal denaturation monitored by the CD signal at 222 nm showed the transition from the native state to the unfolded state of the NR-domain. In reducing conditions, denaturation curves were shifted toward lower temperatures, showing that the protein is destabilised when the disulfide bridge is reduced (figure 4). The values of the melting temperatures (T_m) are shown in table 1: in reducing conditions, T_m values are 10°C lower as in oxidizing conditions. T_m values are similar for $AQ_{24}NR$, $AQ_{12}NR$ and N3 alone showing that this effect on the NR domain is observed independently of the length or the presence of the repetitive region.

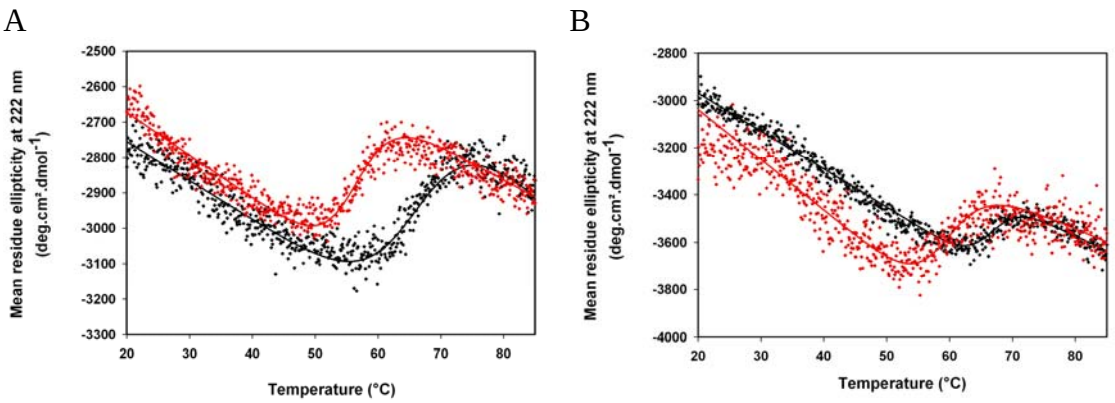

Figure 4. Thermal denaturation of eADF-3 monitored by circular dichroism. A: $AQ_{12}NR$. B: $AQ_{24}NR$. Transitions in oxydizing conditions are in black and in reducing conditions, in red.

Table 1. Melting temperatures determined from eADF-3 thermal denaturations.

	Non reducing conditions	Reducing conditions
AQ12NR	66°C	56°C
AQ24NR	66°C	59°C
NR	64°C	54°C

c) Assembly of eADF-3

Potassium phosphate is an important trigger initiating silk assembly inside a spider's spinning duct (Knight and Vollrath, 2001). eADF-3 aggregation has been investigated upon incubation with various concentrations of potassium phosphate. After two hours of incubation with increasing concentrations of potassium phosphate at 25°C, aggregates were sedimented by centrifugation at 125 000 × g for 30 min and soluble protein remaining in solution was quantified by measurement of UV-absorbance at 276 nm. The percentage of aggregated protein was plotted in function of potassium phosphate concentration (figure 5a). The soluble / insoluble transition occurs at about 200-250 mM of potassium phosphate. No significant difference was observed between $AQ_{24}NR$ and AQ_{24} indicating that the NR domain has no influence on the aggregation of eADF-3. The transition was slightly shifted toward higher potassium phosphate concentrations in the case of $AQ_{12}NR$. In order to check if this difference was due to different lengths of the repetitive domains, aggregation experiments were performed at protein concentrations were the amount of AQ motives was identical: $AQ_{24}NR$ at 7.5 µM and $AQ_{12}NR$ at 15 µM (figure 5b). In that case, the results were indistinguishable, confirming that the length of the repetitive domain is an important factor regulating eADF-3 aggregation.

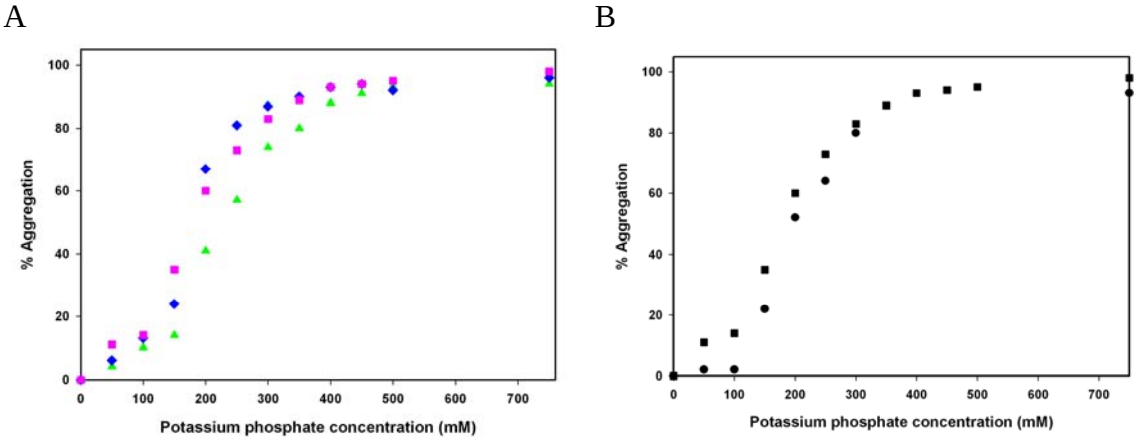

Figure 5. Aggregation of eADF-3 in potassium phosphate at 25°C. A: aggregation of $AQ_{12}NR$ (green triangles), $AQ_{24}NR$ (pink squares) and AQ_{24} (blue diamonds) at 10 µM. B: aggregation of $AQ_{12}NR$ at 15 µM (dots) and $AQ_{24}NR$ at 7.5 µM (squares).

d) Characterization of eADF-3 aggregates

Atomic force microscopy and scanning electron microscopy were used to gain information on the morphology of eADF-3 aggregates. At high phosphate concentrations, eADF-3 assembled into spherical shapes with a diameter of about 1 µm (figure 6). Aggregates formed at low phosphate concentration were hardly identifiable by SEM. However, using AFM, we could observe fibrillar structures of eADF-3 at low potassium phosphate concentrations (figure 7). eADF-3 fibrils showed diameters of about 5 nm.

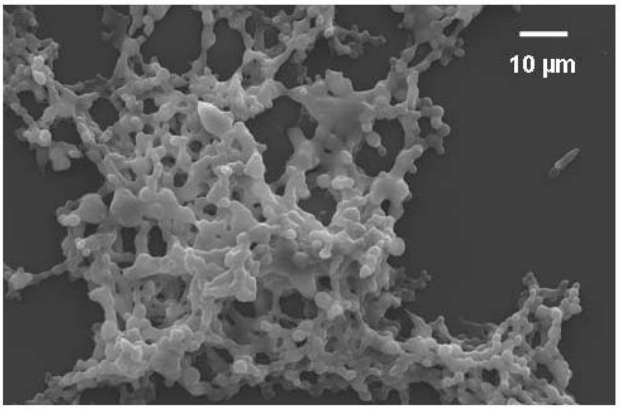

Figure 6. SEM picture of $AQ_{24}NR$ aggregates obtained in 750 mM potassium phosphate.

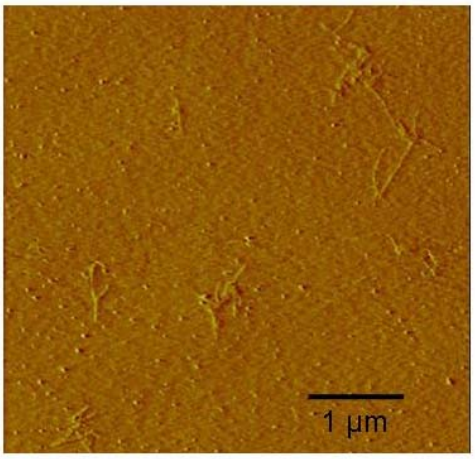

Figure 7. AFM picture of $AQ_{24}NR$ aggregates obtained in 150 mM potassium phosphate.

3) Conclusion

Adf-3 and *adf-4* can be expressed in the cytoplasm of insect cells. Production of rADF-3 and rADF-4 by secretion in cells media increases the solubility of the proteins, especially for rADF-4, arguing that it is likely modified by post-translational modifications

Investigations on eADF-3s produced in *E. coli* showed the importance of the disulfide bridge on the protein stability. Upon incubation with potassium phosphate, eADF-3s are able to

assemble in spherical and fibrillar aggregates. The length of the repetitive domain of the protein seems to be important for aggregation.

Further investigations are necessary to obtain more detailed insights into the assembly mechanism of individual spider silk proteins and to employ spider silk proteins for new materials.

V) Bibliography

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